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# Piperlongumine promotes autophagy via inhibition of Akt/mTOR signalling and mediates cancer cell death

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**Background:** The Akt/mammalian target of rapamycin (mTOR) signalling pathway serves as a critical regulator of cellular growth, proliferation and survival. Akt aberrant activation has been implicated in carcinogenesis and anticancer therapy resistance. Piperlongumine (PL), a natural alkaloid present in the fruit of the Long pepper, is known to exhibit notable anticancer effects. Here we investigate the impact of PL on Akt/mTOR signalling.

**Methods:** We examined Akt/mTOR signalling in cancer cells of various origins including prostate, kidney and breast after PL treatment. Furthermore, cell viability after concomitant treatment with PL and the autophagy inhibitor, Chloroquine (CQ) was assessed. We then examined the efficacy of *in vivo* combination treatment using a mouse xenograft tumour model.

**Results:** We demonstrate for the first time that PL effectively inhibits phosphorylation of Akt target proteins in all tested cells. Furthermore, the downregulation of Akt downstream signalling resulted in decrease of mTORC1 activity and autophagy stimulation. Using the autophagy inhibitor, CQ, the level of PL-induced cellular death was significantly increased. Moreover, concomitant treatment with PL and CQ demonstrated notable antitumour effect in a xenograft mouse model.

**Conclusions:** Our data provide novel therapeutic opportunities to mediate cancer cellular death using PL. As such, PL may afford a novel paradigm for both prevention and treatment of malignancy.

Naturally-occurring substances are the most dependable resource for therapeutic development. A variety of FDA approved anti-neoplastic agents, including docetaxel and topotecan, have demonstrated clinical utility based on ongoing investigations of natural products. Piperlongumine (PL), a natural alkaloid of the Long pepper (*Piper longum*), exhibits numerous key biological activities. In addition to its insecticidal and bactericidal capabilities (Yang *et al*, 2002), recent literature points to the fact that PL is capable of hindering the growth of sarcoma, bladder, breast, melanoma and lung tumours *in vitro* and *in vivo* (Bezerra *et al*, 2008; Raj *et al*, 2011). The ability of PL to target the cellular stress response through direct inhibition of Glutathione S-transferase pi 1 (GSTP1) subsequently inducing of intracellular reactive oxygen

species (ROS) accumulation promoting selective killing of cancer cells was initially demonstrated by Raj *et al* (2011). Furthermore, PL has minimal high-dose acute toxicity, and does not appear to significantly affect any biochemical, haematologic and histopathologic parameters in animal models (Raj *et al*, 2011). In addition, the ability of PL to downregulate NF- $\kappa$ B activation was demonstrated by our and other studies (Han *et al*, 2013). Moreover, PL induces rapid depletion of the androgen receptor (Golovine *et al*, 2013) in prostate cancer cells.

The serine/threonine kinase Akt functions as a critical control point of multiple upstream signalling pathways involving receptor tyrosine kinase stimulation. Activation of Akt promotes cellular growth and proliferation as well as inhibition of apoptosis

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(Majewski *et al*, 2004). Akt aberrant activation has been implicated in the development of malignancy and anticancer therapy resistance (Gutierrez *et al*, 2011; Opel *et al*, 2011).

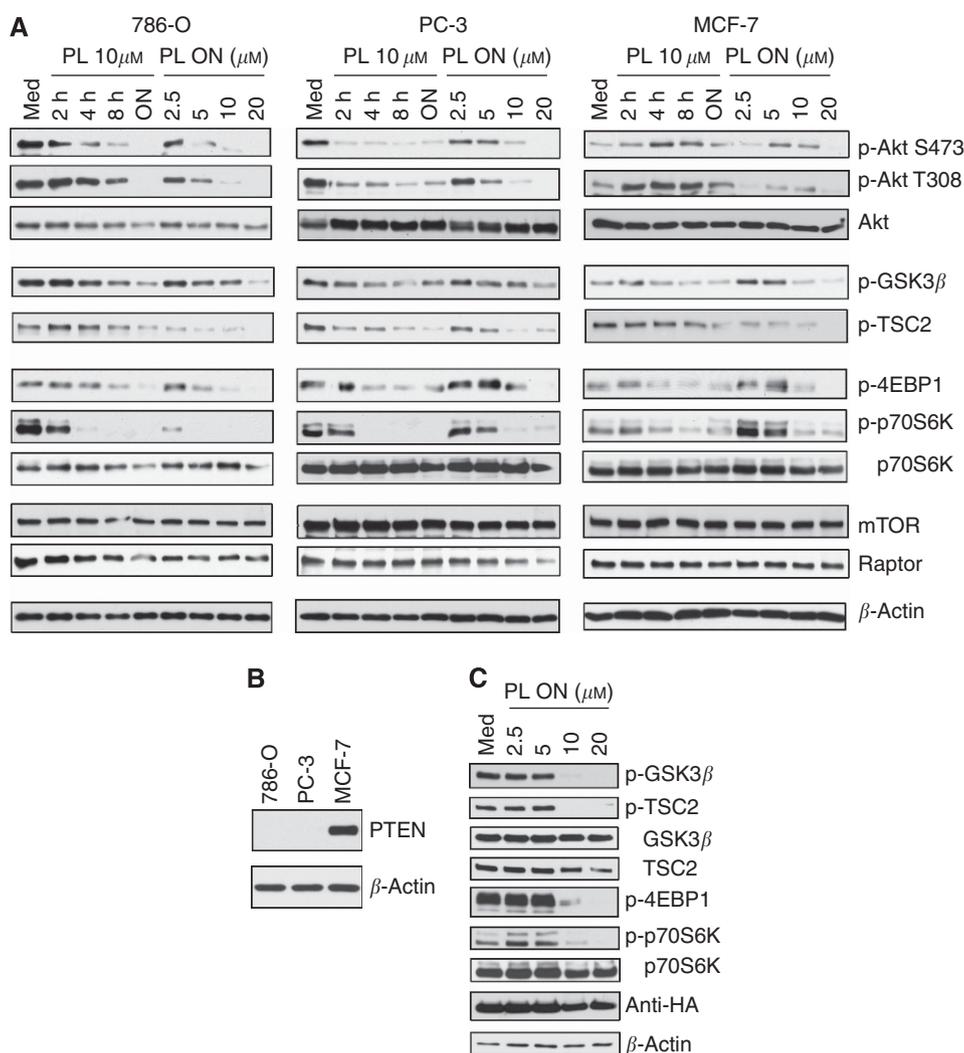
An effector of Akt functioning is the mammalian target of rapamycin (mTOR), a serine/threonine kinase. A number of recent publications dealing with mechanistic actions of mTORC1 complex provide clear evidence that in addition to its well-established effects it also acts as a key checkpoint in signalling pathways regulating autophagy (Alers *et al*, 2011). Autophagy has been characterised as critical homeostatic and adaptive process that serves to deliver cytoplasmic proteins and organelles to the lysosomes for digestion.

The overall clinical picture, however, is not well defined. Studies of autophagy in different cell types under a spectrum of various cellular conditions provide conflicting evidence regarding its role in cellular death (Debnath *et al*, 2005; Levine and Kroemer, 2008; White and DiPaola, 2009). Whereas the inhibition of autophagy appears to enhance the sensitivity of tumour cells towards anti-neoplastic drugs (Amaravadi *et al*, 2007), the induction of autophagy may promote tumour cell death in those with heightened resistance to apoptosis.

In our present study, we provide first evidence that PL is capable of stimulating autophagy in various cancer cell lines, including prostate, kidney and breast. It does so via ROS-dependent direct downregulation of Akt/mTOR signalling, subsequently promoting increased autophagy in the aforementioned cells. Additionally using autophagy inhibitor, Chloroquine (CQ), we were able to modulate PL-induced cellular death. Furthermore, CQ significantly increased antitumor activity of PL in the xenograft mouse model. Taken together, our data provide novel pathways to potentially mediate cancer cell death by PL-induced autophagy.

## MATERIALS AND METHODS

**Cells and culture conditions.** Androgen-independent human PC-3 prostate cancer cells, breast cancer MCF-7 cells, renal carcinoma 786-O cells and HEK-293T kidney cells were obtained from ATCC (Rockville, MD, USA). Cells were cultured in RPMI 1640 (Bio-Whittaker, Walkersville, MD, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), penicillin (100 U ml<sup>-1</sup>), streptomycin



**Figure 1.** Piperlongumine affects Akt downstream signalling in cancer cells of various origins. **(A)** Piperlongumine decreases phosphorylation levels of Akt effectors, GSK-3 $\beta$  and TSC2. Additionally, PL treatment results in significant downregulation of mTORC1 complex activity but did not affect the main components of mTORC1 complex, mTOR and Raptor. Cells were treated with PL at indicated concentrations for 2, 4, 8 h or overnight (ON, 20–24 h). **(B)** Western blot analysis of PTEN expression in 786-O, PC-3 and MCF-7 cells. **(C)** The inhibitory effect of PL on functioning of constitutively active Akt(T308D; S473D) mutant in MCF-7 cells. MCF-7-HA-Akt<sup>mut</sup> cells were treated with PL at indicated concentrations for 24 h. Total cellular lysates were subjected to western blotting with the specific antibodies.

(100  $\mu\text{g ml}^{-1}$ ), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM) under conditions indicated in the figure legends.

**Antibodies and reagents.** Antibodies to Akt, phospho-Akt(S473), phospho-Akt(T308), phospho-Tuberin/TSC2(T1462), Tuberin/TSC2, GSK-3 $\beta$ , phospho-GSK-3 $\beta$ (S9), mTOR, Raptor, phospho-4E-BP1(T37/46), p70S6K, phospho-p70S6K(T389), LC3A/B, ULK1, phospho-ULK1(S757) and Anti-Rabbit IgG Fab2 Alexa Fluor (R) 488 conjugate were obtained from Cell Signaling Technology (Beverly, MA, USA).  $\beta$ -Actin and HA-probe antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Piperlongumine was obtained from Indofine Chemical Company (Hillsborough, NJ, USA). Chloroquine diphosphate, Puromycin dihydrochloride, Hexadimethrine bromide and N-Acetyl-L-Cysteine were obtained from Sigma-Aldrich (St Louis, MO, USA). Bafilomycin A1 was obtained from LC Laboratories (Woburn, MA, USA).

**Western blot analysis.** Whole-cell lysates preparation and western blot analysis were performed as described previously (Golovine *et al*, 2008).

Analysis of intracellular ROS production was performed as described previously (Golovine *et al*, 2013).

**Establishment of PL ED50 for cancer cell lines.** MCF-7 and 786-O cells were incubated with various PL concentrations for 48 h. Cell proliferation was analysed by CellTiter Blue assay (Promega, Madison, WI, USA). Effective doses (ED) were calculated using XLift, Microsoft Excel add-in.

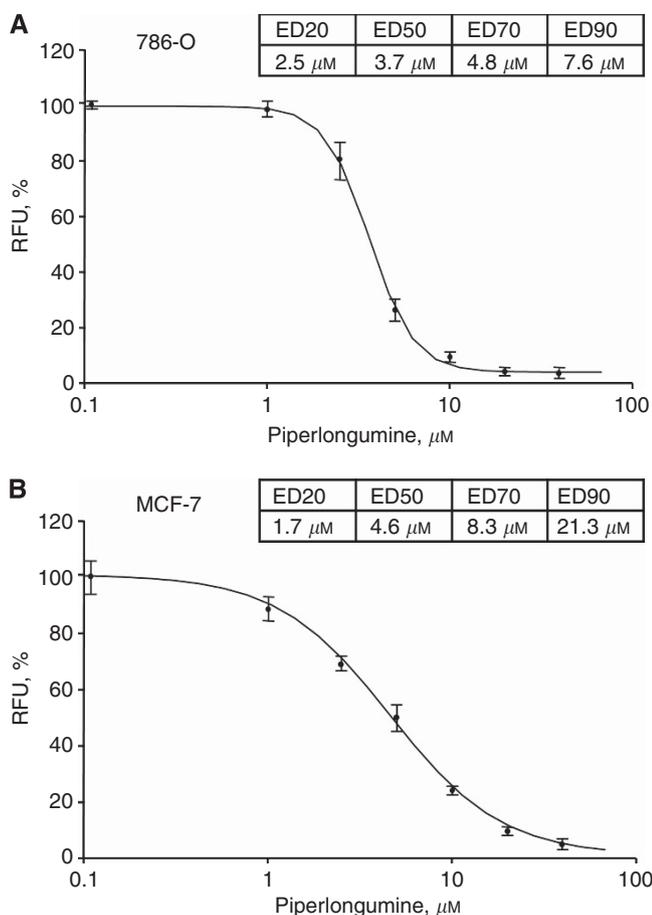


Figure 2. Piperlongumine demonstrates antiproliferative effect in 786-O (A) and MCF-7 (B) cells. Cells were treated with indicated concentrations of PL for 48 h. Cellular proliferation was assessed using the CellTiter Blue assay.

**Generation of MCF-7 cell line stably expressing constitutively active Akt.** The mammalian expression vector pcDNA3 (plasmid 14751) encoding HA-tagged constitutively active Akt(T308D; S473D) mutant was obtained from Addgene (Cambridge, MA, USA). HA-Akt<sup>mut</sup> ORF was then recloned into pLV-CMV-H4-puro lentiviral construct. HEK-293T cells were concomitantly transfected with plasmids, pLV-CMV-HA-Akt<sup>mut</sup>-H4-puro, pCMVdeltaR8.2 and pVSV-G (Clontech, Mountain View, CA, USA) using TransIT-293 Transfection reagent (Mirus, Madison, WI, USA). pLV-CMV-H4-puro and pCMVdeltaR8.2 constructs were kindly provided by Dr Alexey Ivanov (West Virginia University School of Medicine, Morgantown, WV, USA). Supernatant of HEK-293T cells containing lentiviral particles was collected 48 h after transfection, filtered through 0.22  $\mu\text{m}$  PVDF syringe filters and used for the infection of MCF-7 cells in presence of 4  $\mu\text{g ml}^{-1}$  Polybrene (Hexadimethrine bromide). Forty-eight hours after infection, puromycin (1  $\mu\text{g ml}^{-1}$ ) was added to the cellular media for further selection of infected cells.

**Assessment of autophagy puncta formation by immunofluorescence.** Immunofluorescent staining of LC3-II protein was carried out according to the protocol available at the official web site of Cell Signaling Technology ([http://www.cellsignal.com/support/protocols/IF\\_methanol.html](http://www.cellsignal.com/support/protocols/IF_methanol.html)). Confocal imaging was performed at room temperature using Nikon laser confocal microscope (model C1). Images were acquired using  $\times 60$  oil lens using the EZ-C1 software package.

**Analysis of cell death.** The evaluation of cell death was performed using Propidium iodide (PI) staining with following Flow Cytometry analysis. Analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Individual fluorescent populations were determined through the use of acquisition and analysis software (Cell Quest, Becton Dickinson).

**Assessment of *in vivo* tumour growth.** For *in vivo* studies,  $1 \times 10^6$  PC-3 cells were inoculated s.c. in the flank region of 6-week-old male C.B17/Icr-scid mice using a 27-gauge needle. All animal procedures were done in accordance with the institutional guidelines on animal care and with appropriate institutional certification. Animals were fed an autoclaved AIN-93M diet (Harlan Teklad, Madison, WI, USA) and water *ad libitum*. Ten days after the injection of tumour cells, animals were randomly

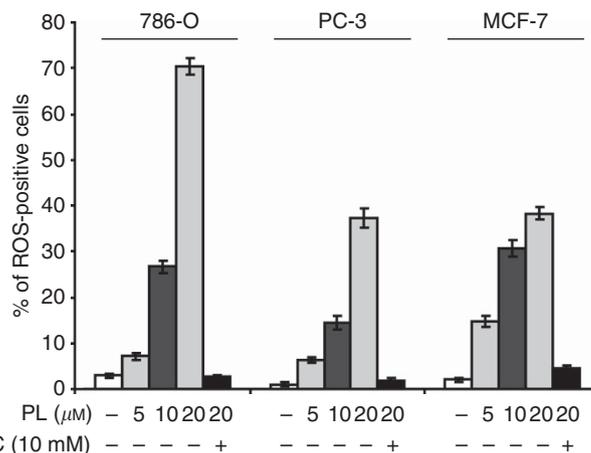


Figure 3. Treatment of 786-O, PC-3 and MCF-7 cells with PL results in increased intracellular ROS production, whereas addition of NAC completely blocks this process. Cells were treated with PL and/or NAC for 2 h. Harvested cells were stained with CM-H<sub>2</sub>DCFDA and analysed by flow cytometry. The figure shows the representative data of one of three separate experiments (Bars, s.e.m. of triplicates).

assigned to the control or experimental groups ( $n=5$  mice per group). The mice were treated with (i) 0.9% NaCl, 0.3% DMSO solution (vehicle); (ii) PL (20 mg kg<sup>-1</sup> in 0.9% NaCl, 0.3% DMSO solution, daily, I.P.); (iii) CQ (40 mg kg<sup>-1</sup>, in 0.9% NaCl, 0.3% DMSO solution daily, I.P.); or (iv) with PL and CQ combined. Tumours were measured twice weekly and their volumes were calculated using the formula: (volume = 0.52 × (width)<sup>2</sup> × length).

## RESULTS

**Piperlongumine decreases the phosphorylation level of Akt and/or its downstream effectors.** Recent publication reported the ability of PL to increase apoptotic cell death in cancer cells while exerting profound antitumour effects *in vitro* and *in vivo* (Raj *et al*, 2011). Taking into consideration that Akt promotes anti-apoptotic and pro-tumourigenic activities, we first turned our attention towards the investigation of the effects of PL on Akt downstream signalling. Three distinct organ cancer cell lines, prostate (PC-3), kidney (786-O) and breast (MCF-7) were chosen for the present study. The treatment of cells with PL resulted in a dramatic decrease of Akt/mTOR signalling. The phosphorylation levels of Akt downstream effectors, GSK-3 $\beta$  and TSC2, were notably depressed in all tested cell lines (Figure 1A). Moreover, PL-induced inhibition of Akt resulted in significant decrease of the mTORC1 complex activity, as made evident by the lowered phosphorylation levels of mTORC1 effectors, 4E-BP1 and p70S6K. Piperlongumine effects were uniformly time- and dose-dependent.

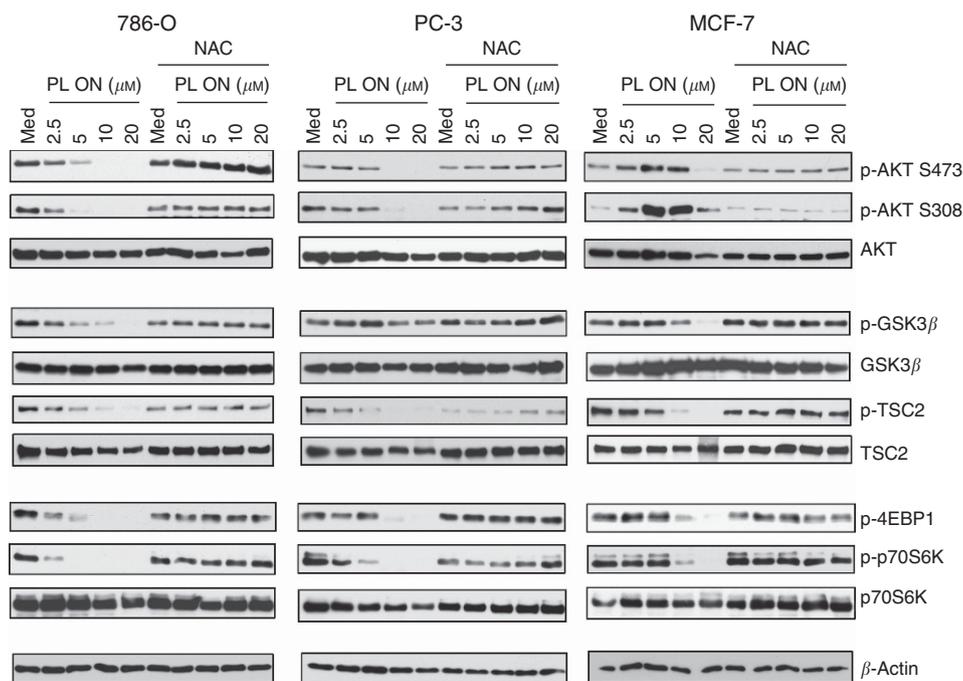
Notably, examination of the T308 and S473 phosphorylation levels of Akt in PL-treated cells yielded an alternate result. PC-3 and 786-O PL-treated cells exhibited decrease in phosphorylation levels both in time- and dose-dependent observations. Furthermore, PL-treated MCF-7 cells demonstrated a paradoxical increase T308 and S473 phosphorylation levels of Akt. This effect was reversed at concentration of 20  $\mu$ M, suggesting that PL acts in a non-specific and toxic manner at those levels.

Regardless of the impact on phosphorylation of Akt, PL attenuated the phosphorylation of downstream Akt targets. Taken together, our data provide evidence that various downstream Akt effectors may be affected by PL administration, PL affects Akt phosphorylation in a cell-specific manner dependently on phosphatase and tensin homologue (PTEN) expression (Figure 1B); however, phosphorylation of Akt effectors was inhibited uniformly.

To further elucidate the effect of PL on Akt downstream targets and its impact on the upstream activation of Akt, we generated a stably expressing, HA-tagged, constitutively active Akt(T308D; S473D) mutant MCF-7 cell line (MCF-7-HA-Akt<sup>mut</sup>). MCF-7-HA-Akt<sup>mut</sup> cells were treated with various concentrations of PL for a 24 h period. Predictably, PL treatment resulted in significant downregulation of GSK-3 $\beta$  and TSC2 phosphorylation levels. When administered at 10  $\mu$ M or higher, PL demonstrated an observable impact on downstream Akt effectors (Figure 1C). Additionally, PL-treated MCF-7-HA-Akt<sup>mut</sup> cells showed a marked decrease in the mTORC1 activity level.

**Cellular proliferation is affected by PL.** 786-O and MCF-7 cell lines were administered with varying concentrations of PL to evaluate PL IC<sub>50</sub>. Relatively to PL mediated decrease of Akt effectors phosphorylation levels, PL affected cellular proliferation, dose-dependently (Figure 2). Similar results demonstrating that PL effectively blocks proliferation of PC-3 cells have been published recently (Ginzburg *et al*, 2013).

**Elevation of ROS levels as a result of PL treatment is responsible for Akt inactivation.** To examine whether PL affects the phosphorylation level of Akt effectors via the ROS pathway, we measured the level of intracellular ROS following treatment with PL. Cells were treated with increasing concentrations of PL alone or concomitantly with a well-established antioxidant, N-Acetyl-L-Cysteine (NAC). Expectedly, the increase in ROS production in PL-treated cells was observed in a dose-dependent manner and was blocked by the addition of NAC to the cellular



**Figure 4.** NAC reverses negative effects of PL on Akt downstream signalling. 786-O, PC-3 and MCF-7 cells were treated with PL at indicated concentrations alone or in combination with 10 mM of NAC for 24 h. Total cellular lysates were subjected to western blotting with specific antibodies.

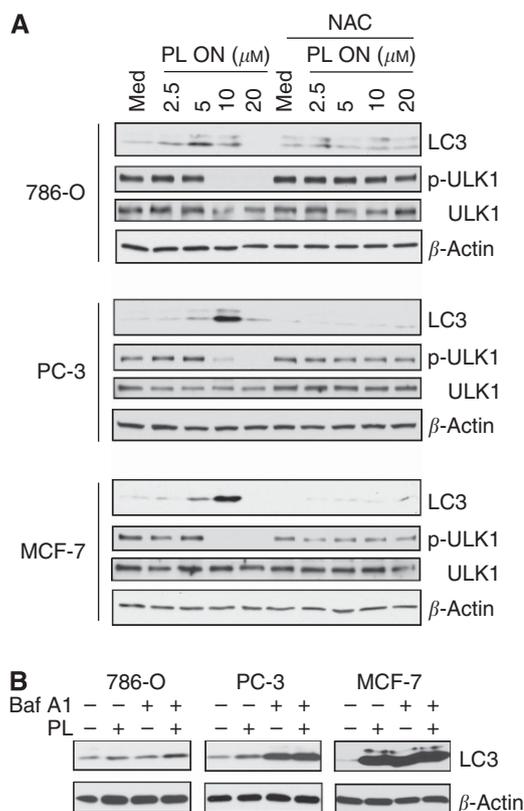
medium (Figure 3). Furthermore, NAC administration completely reversed PL-induced Akt functional changes in every tested cell line. Figure 4 illustrates that PL-associated causes decrease in phosphorylation levels of Akt effectors, GSK-3 $\beta$  and TSC2, and mTORC1 target proteins, 4E-BP1 and p70S6K, were abolished in cells treated concomitantly with NAC. Treatment with NAC alone did not induce any changes in either phospho-GSK-3 $\beta$  and phospho-TSC2 protein levels, or in the phosphorylated forms of 4E-BP1 and p70S6K. Moreover, cells treated with excessive amounts of PL (20  $\mu$ M) were able to overcome its toxic effects following reversal with NAC. Our experimental data offer compelling evidence that PL exerts a strong negative effect on Akt downstream signalling through indirect stimulation of ROS.

**Piperlongumine-induced inactivation of Akt/mTORC1 signalling promotes autophagy.** After our initial data demonstrated the inhibitory effects of PL on the Akt/mTORC1 pathway, we extended our analysis to examine the role played by PL in the process of autophagy. Cells were treated with increased concentrations of PL alone or in presence of NAC (10 mM) for 24 h. The microtubule-associated protein 1 light chain 3 (LC3) is widely used as a marker for autophagy (Tanida *et al*, 2004). Therefore, we performed western blot analysis utilising anti-LC3A/B antibodies that preferentially bind LC3-II protein. We observed that LC3-II protein accumulation in all tested cells responded to the administration of PL (Figure 5A). Treatment with excessive amounts of PL (20  $\mu$ M) resulted in undetectable levels of LC3-II, further offering evidence of the deleterious/toxic effect of PL at high concentrations. Piperlongumine-mediated autophagy in all tested cell lines was ROS-dependent, made evident by the complete reversal of LC3-II accumulation (Figure 5A) and mTORC1 inhibition following the administration of NAC.

As ULK1, a mammalian autophagy-initiating kinase is directly controlled by mTORC1 (Kim *et al*, 2011), we additionally examined whether ULK1 serine 757 phosphorylation levels would be affected by PL treatment. Expectedly, PL treatment resulted in dramatic downregulation of the phospho-Ser757 ULK1 levels in all tested cell lines (Figure 5A).

Increased LC3-II levels may be observed during enhanced autophagosome formation or reduced autophagosome turnover (Rubinsztein *et al*, 2009). To further substantiate our results, we examined level changes of processed LC3-II in cells treated with PL alone and PL with a well-established autophagy inhibitor, Bafilomycin A1 (Baf-A1). Our data indicate that cells treated concomitantly with Baf-A1 and PL accumulate LC3-II at higher levels, further supporting PL's role as an autophagy inducer (Figure 5B). Data were further confirmed through the analysis of autophagosome formation by immunofluorescence using anti-LC3A/B antibodies preferentially binding the type II form of LC3A/B. Providing further validation to our initial results, LC3 punctum formation was observed by fluorescent microscopy solely in cells treated with PL and temsirolimus (positive control) (Figure 6). Cells treated with concomitant PL and NAC showed a barely detectable LC3 punctum formation, mimicking untreated cells displaying basal autophagy levels.

**Modulation of PL-mediated cell death by autophagy inhibitors.** Autophagy promotes the survival of cells resistant to apoptosis when they are deprived of extracellular nutrients or growth factors and represents a mechanism of resistance to anticancer therapy-mediated cell death (Dikic *et al*, 2010). With that in mind, we turned our attention towards examining the effect of autophagy inhibition on PL-induced cell death. We utilised an established autophagy inhibitor, CQ, which has been shown to exhibit antitumour effects and sensitise cancer cells to chemotherapeutic drugs (Amaravadi *et al*, 2007; Firat *et al*,



**Figure 5.** Treatment with PL promotes autophagy induction. (A) Piperlongumine treatment results in LC3-II accumulation and decrease of ULK1 Ser757 phosphorylation. Piperlongumine induction of autophagy is ROS dependent, as NAC completely reverses effects of PL. Cells were treated with indicated concentrations of PL alone or in combination with 10 mM of NAC for 24 h. (B) Concomitant treatment of cells with PL (10  $\mu$ M) and Baf-A1 (10  $\mu$ M) results in higher accumulation of LC3-II then treatment with PL alone, which indicates PL acts as autophagy inducer rather than inhibitor. Total cellular lysates were subjected to western blotting with specific antibodies. Upper band (if visible) corresponds to LC3-I and lower band corresponds to LC3-II form of LC3 protein.

2012). Cells were treated with either 20  $\mu$ M of CQ alone, with 10  $\mu$ M of PL alone or concomitantly for 72 h. Treatment with PL alone led to a measurable induction of cell death, whereas concomitant treatment with PL and CQ resulted in the most profound measured levels of cell death in all tested cell lines (Figure 7). These results bring forth a critical point that PL-induced cellular death can be enhanced through concurrent autophagy inhibition, CQ in this case.

**Antitumour effects of PL enhanced by CQ *in vivo*.** Our data presented above clearly demonstrate the ability of CQ to sensitise cancer cells to PL *in vitro*. We next, extended our findings by evaluation of antitumour effects of PL alone or in combination with CQ *in vivo*. Xenograft tumours were established in severe combined immunodeficient mice using PC-3 cells. Animals were administered CQ (40 mg kg<sup>-1</sup>) or PL (20 mg kg<sup>-1</sup>) daily through intraperitoneal injections as monotherapy, or as combination therapy at indicated doses. As demonstrated in Figure 8, treatment with CQ alone did not yield any significant tumour regression. Treatment with PL alone resulted in a statistically significant inhibition of tumour growth. Concomitant treatment with PL and CQ, however, resulted in the most profound regression of tumour mass.

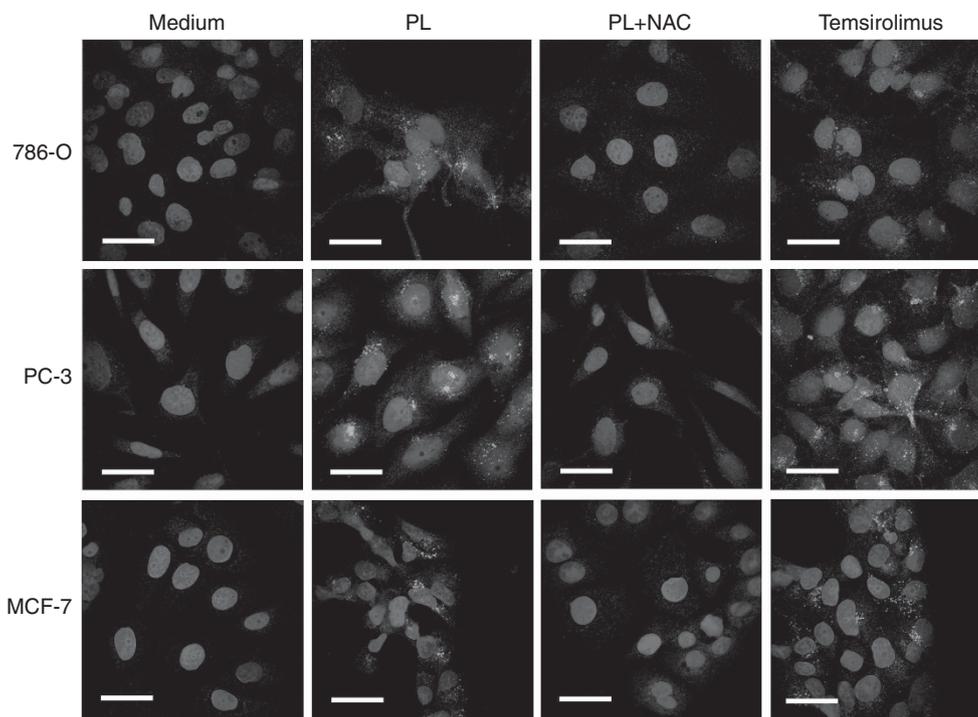


Figure 6. Immunofluorescent detection of increased autophagosome flux in cells treated with PL. N-Acetyl-L-Cysteine reverses the autophagy-inducing effect of PL. Additionally, cells were treated with mTORC1 inhibitor, temsirolimus, which induced autophagy serving as a positive control. Light chain 3-II is shown in green and DAPI in blue. Bar, 50  $\mu\text{m}$ . The full colour version of this figure is available at *British Journal of Cancer* online.

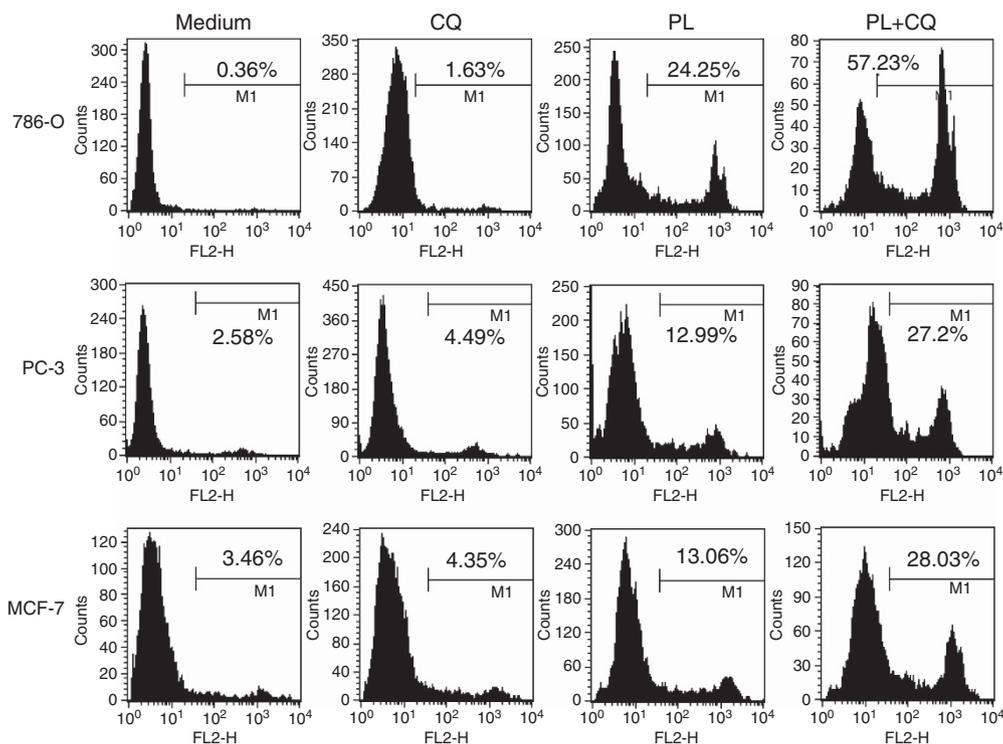
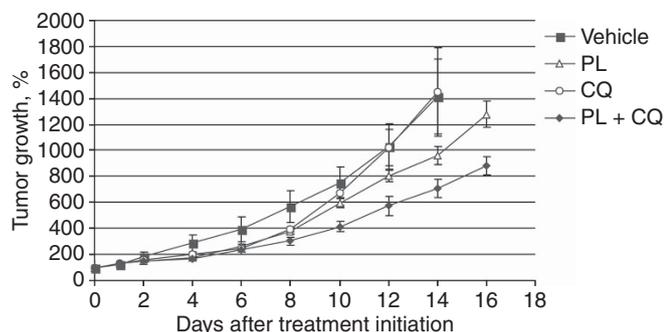


Figure 7. Inhibition of autophagy by CQ promotes PL-mediated cancer cell death *in vitro*. Cells were treated with either 20  $\mu\text{M}$  of CQ alone, with 10  $\mu\text{M}$  of PL alone or concomitantly for 72 h. Cells were then harvested, PI was added to cellular suspensions at 3  $\mu\text{g ml}^{-1}$  concentration and analysed by Flow cytometry. The representative data from one of three independent experiments are presented.



**Figure 8.** The concomitant treatment with PL and CQ results in inhibition of tumour growth xenograft mouse tumour model. Subcutaneous PC-3 tumors were established in 6-week-old male C B17/lcr-scid mice. Treatment with PL and/or CQ and assessment of tumor growth were carried out as described in Materials and Methods. Data shown are mean of five mice in each group (s.e.m. displayed with bars).

## DISCUSSION

Physiologically ROS are toxic by-products that are generated by the mitochondria via a multicomponent NADPH oxidase enzymatic complex of the respiratory chain (Balaban *et al*, 2005). To date, compelling evidence exists that points to ROS function as an important physiological regulator of intracellular signalling pathways (Ray *et al*, 2012).

Recent publications reveal the antitumour role of ROS, which is carried out through several distinct mechanisms. Reactive oxygen species has been linked to mediation of apoptosis through activation of JNK signalling (Whibley *et al*, 2007). In addition, recent work published by Raj *et al* (2011) demonstrates direct involvement of ROS in selective killing of cancer cells.

The Akt/mTOR signalling pathway has a crucial regulatory role in cellular proliferation and survival, glucose metabolism and angiogenesis (Manning and Cantley, 2007). A host of recent publications deal with the impact of ROS on Akt/mTOR signalling. Enhanced Akt signalling primarily via the ROS-mediated inactivation of PTEN has been well documented in multiple reports (Leslie, 2006; Yalcin *et al*, 2010; Shearn *et al*, 2011b). Other data elaborate that in addition to its positive modulating effect on Akt signalling, ROS is capable of exerting a direct target effect on Akt itself under conditions of oxidative stress (Murata *et al*, 2003; Hussain *et al*, 2011; Shearn *et al*, 2011a).

Our current work declares that PL-mediated ROS generation promotes an inhibitory response on Akt/mTOR signalling and is involved in autophagy induction. Indeed, we observed a dramatic effect on phosphorylation of Akt effectors across all tested cancer cell lines, following administration of PL. As an added validity to our hypothesis that PL inhibition of Akt/mTOR signalling is mediated by ROS, administration of a well-established antioxidant, NAC completely reversed all cytotoxic effects of PL.

In our results, we point out the diverse effects of PL on phosphorylation levels of S473 and T308 Akt sites. This is likely explained by cellular PTEN expression status and consistent with prior studies demonstrating inactivation of PTEN by ROS (Leslie, 2006). Furthermore, a strong possibility of positive feedback exists (Sun *et al*, 2005; O'Reilly *et al*, 2006), which explains the downregulation of mTORC1 activity in response to the inhibition of downstream Akt signalling. Indeed, when PTEN-positive MCF-7 cells were treated with PL, we observed enhanced Akt phosphorylation. Similar results were obtained when PTEN-positive cell lines of other origins, DU-145 (prostate cancer) and 769-P (kidney cancer) were treated with PL (data not presented). However, it would be hard to clarify whether PL has a direct

inhibitory effect on mTOR kinase activity, or it may impair the mTORC1 complex integrity, or it may even affect other members of mTORC1 complex and dissect the data of mTORC1 itself functioning from dependence on Akt activity.

Phosphatase and tensin homologue-negative PC-3 and 786-O cells exhibit an independently high level of Akt phosphorylation even in the absence of strong upstream stimulation, which is necessary for overcoming the inhibitory role of PTEN (Ramawamy *et al*, 1999). Interestingly, our results demonstrated the decrease of Akt S473 phosphorylation in PTEN-negative PC-3 and 786-O cells, indicating the potential possibility of PL ability to downregulate mTORC2 activity. However, it is difficult to clarify whether PL reduces the activity of mTORC2 complex, or PL reduces its upstream activation. Here we speculate that in PTEN-negative cells, PL acts as a negative regulator of Akt; most likely through decrease in the upstream stimulation of mTORC2 complex rather than directly effecting mTORC2. This speculation is supported by the following observations: (a) PL treatment of PTEN-negative PC-3 and 786-O cells resulted in decrease in phosphorylation levels of both, p-Akt(T307) and p-Akt(S473); (b) PL did not show any negative effects on mTORC2 complex activity in PTEN-positive MCF-7 cells. Indeed, PTEN-negative cells are more sensitive to stimulation by growth factors (Sun *et al*, 1999) and upstream activation. Although PL-treated PTEN-positive MCF-7 cells demonstrate an associated rise in phospho-Akt levels, our data point out that Akt activity is in fact inhibited via a direct ROS-mediated effect. Piperlongumine-treated MCF-7 cells expressing constitutively active Akt revealed a similar decrease in phosphorylation level of Akt targets as in parental cells.

The role of the mTORC1 complex as a positive regulator of protein synthesis, cell growth and proliferation has been well established (Fingar and Blenis, 2004; Wang *et al*, 2005). Recent studies have shown that treatment with H<sub>2</sub>O<sub>2</sub> and other oxidative agents increases the activation of Akt/mTOR signalling (Sarbasov and Sabatini, 2005; Li *et al*, 2010; Yoshida *et al*, 2011), whereas high doses of these agents have the reverse effect on mTORC1 activity (Kim *et al*, 2002; Liu *et al*, 2008; Li *et al*, 2010). Our data are in concert with the recently published data regarding negative redox regulation of mTORC1. Our group provides firsthand evidence that PL mediates mTORC1 inhibition via an Akt-dependent mechanism.

Mammalian target of rapamycin complex 1 is also known for its ability to halt cellular catabolic processes such as autophagy (Kim *et al*, 2011). Expectedly, PL-mediated inhibition of mTORC1 resulted in autophagy induction in all tested cell lines, as demonstrated by the accumulation of LC3-II and decrease of serine 757 phosphorylation levels of ULK1 in PL-treated cells. In addition, our LC3-II accumulation assay was further reinforced by detection of autophagic puncta in cells treated with PL by immunofluorescence.

The link between autophagy and carcinogenesis has been well examined. Autophagy may provide cancer cells additional sources of energy and nutrients during periods of rapid tumour growth. Autophagy has been considered by some as type II programmed cell death, a concept which fell under heavy debate in recent years. The Nomenclature Committee of Cell Death 2009 points out that the term 'may misleadingly suggest a form of death occurring through autophagy, as this process often promotes cell survival' (Galluzzi *et al*, 2009). Presently recognised as a promoter of cancer cell survival, autophagy has become a target for the development of alternate anti-neoplastic strategies (Amaravadi *et al*, 2007; Apel *et al*, 2008; Palacios *et al*, 2010; Wu *et al*, 2010; Amaravadi *et al*, 2011). Our results further elaborate on the previously published data, providing clear evidence that inhibition of autophagy facilitates cancer cell death in response to anticancer therapy. Through our application of a well-established autophagy inhibitor, CQ, we were able to significantly increase the level of cancer cell

death when treated with PL. As added proof of principle, concomitant treatment with PL and CQ resulted in inhibition of xenograft model tumour growth.

Our work brings forth a number of key new concepts in an attempt to better understand the cellular alterations that take place following administration of PL. We provide first evidence that PL capably reduces the activity of Akt/mTOR signalling through generation of ROS, the effects of which are blocked by the administration of NAC. Furthermore, PL-induced Akt inactivation reduces mTORC1 activity subsequently promoting cell autophagy. In consistence with a number of recent data reporting cooperative inhibition of autophagy and PI3K/Akt/mTORC1 signalling pathway result in sensitization of cancer cells to the therapeutic stimuli (Degtyarev *et al*, 2008; Fan *et al*, 2010; Firat *et al*, 2012; Xie *et al*, 2013), our data provide strong support for the development of novel therapeutic strategies based on concomitant use of autophagy inhibitors and PL. This is a crucial concept, as it opens potential clinical avenues of modulating PL-induced cellular death through inhibition of autophagy.

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